

Research Article

# The Regulation of Circulating Hepatokines by Fructose Ingestion in Humans

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**Abbreviations:** ANGPTL4, angiotensin-like 4; ANOVA, analysis of variance; BMI, body mass index; cAMP, cyclic adenosine monophosphate; ChREBP, carbohydrate-responsive element binding protein; ELISA, enzyme-linked immunosorbent assay; FGF21, fibroblast growth factor 21; FoxO, forkhead box O; GDF15, growth differentiation factor 15; LDL, low-density lipoprotein; PPAR, peroxisome proliferator-activated receptor.

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## Abstract

**Context:** Fibroblast growth factor 21 (FGF21), follistatin, angiotensin-like 4 (ANGPTL4), and growth differentiation factor 15 (GDF15) are regulated by energy metabolism. Recent findings in humans demonstrate that fructose ingestion increases circulating FGF21, with increased response in conditions of insulin resistance.

**Objective:** This study examines the acute effect of fructose and somatostatin on circulating FGF21, follistatin, ANGPTL4, and GDF15 in humans.

**Methods:** Plasma FGF21, follistatin, ANGPTL4, and GDF15 concentrations were measured in response to oral ingestion of 75 g of fructose in 10 young healthy males with and without a 15-minute infusion of somatostatin to block insulin secretion. A control infusion of somatostatin was also performed in the same subjects.

**Results:** Following fructose ingestion, plasma FGF21 peaked at 3.7-fold higher than basal concentration ( $P < 0.05$ ), and it increased 4.9-fold compared with basal concentration ( $P < 0.05$ ) when somatostatin was infused. Plasma follistatin increased 1.8-fold after fructose ingestion ( $P < 0.05$ ), but this increase was blunted by concomitant somatostatin infusion. For plasma ANGPTL4 and GDF15, no increases were obtained following fructose ingestion. Infusion of somatostatin alone slightly increased plasma FGF21 and follistatin.

**Conclusion:** Here we show that in humans (1) the fructose-induced increase in plasma FGF21 was enhanced when somatostatin was infused, suggesting an inhibitory role of insulin on the fructose-induced FGF21 increase; (2) fructose ingestion also increased plasma follistatin, but somatostatin infusion blunted the increase; and (3) fructose ingestion had no stimulating effect on ANGPTL4 and GDF15 levels, demonstrating differences in the hepatokine response to fructose ingestion.

**Key Words:** FGF21, follistatin, ANGPTL4, GDF15, insulin resistance, glucagon

A novel aspect of the liver is the secretion of signaling molecules—termed hepatokines—implicated in metabolism. The hepatokine secretion is particularly regulated by metabolic stressful conditions, either physiological (such as fasting or exercise) or pathophysiological (such as obesity and insulin resistance) [1]. Likewise, increased fructose consumption has been linked to metabolic diseases, including obesity, type 2 diabetes and nonalcoholic fatty liver disease; however, the link remains controversial [2-5]. Recently, it has been shown that fructose ingestion increases circulating levels of the hepatokine fibroblast growth factor 21 (FGF21), with a higher response in subjects with metabolic disease compared with healthy subjects [6], supporting a link between hepatokines, fructose, and metabolic disease.

FGF21 was identified as a fasting-induced liver-derived signaling molecule [7-9] that ameliorates insulin resistance and reduces low-density lipoprotein (LDL)-cholesterol, body weight and triglycerides in humans [10]. Other signaling molecules, including follistatin and angiopoietin-like 4 (ANGPTL4), were later demonstrated to be liver-derived in humans [11, 12] and implicated in metabolism [13-16]. Increased circulating levels of hepatokines have been associated with conditions of dysregulated energy metabolism and insulin resistance. Circulating levels of FGF21 are increased in humans during critical illness [17] and with conditions such as obesity [18], type 2 diabetes [19], steatosis [18] and nonalcoholic steatohepatitis [18]. Likewise, an increase in circulating levels of follistatin has been shown in patients with type 2 diabetes [20], during critical illness [21, 22] and during pregnancy [23]. For ANGPTL4, increased circulating levels have been shown in humans with obesity and glucose intolerance [24]. Thus, the understanding of how hepatokines are regulated in humans is only emerging.

The 2 signalling pathways that have been identified as increasing secretion of hepatokines are activation of the peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) and the glucagon pathway. Insulin has an inhibitory effect on hepatokine secretion, both in vivo and in vitro [11, 12, 25]. Forkhead Box O (FoxO) signaling has been pinpointed as an intracellular pathway of insulin-inhibited follistatin secretion [26].

In parallel with glucagon, circulating FGF21 also increases acutely following fructose ingestion [6]. Furthermore, higher basal and fructose-stimulated FGF21 levels were associated with elevated basal endogenous glucose production, basal lipolysis, and peripheral insulin resistance, which all are well-defined features of metabolic disease [27]. Therefore, it could be speculated that reduced insulin action increases the fructose-induced FGF21 response as observed during glucagon stimulation [11, 12, 25]. Whether the other glucagon-regulated

hepatokines—follistatin and ANGPTL4—are regulated by fructose ingestion is not known.

The hypothesis of the present study was that fructose-induced FGF21 secretion is inhibited by circulating insulin as observed for the glucagon-induced FGF21 response, and furthermore to investigate whether follistatin and ANGPTL4—both established hepatokines—are regulated in a similar manner as FGF21 by fructose. Finally, growth differential factor 15 (GDF15) is similar to FGF21 in many respects. Both are primarily expressed in the liver [9, 28], increased in the circulation in response to an acute bout of exercise [25, 29], and found elevated with type 2 diabetes [30-32]. For secretion of GDF15, both liver [33] and intestinal [34] origin has been proposed, when human subjects were treated with the anti-diabetic drug metformin. As GDF15 shares several similarities with the established hepatokines and fructose impacts both the intestine and liver, GDF15 was included in the study.

## Methods

### Subjects

Ten healthy young men were recruited for the study. Inclusion criteria were: men from 18 to 30 years of age, body mass index (BMI) 20 to 25 kg/m<sup>2</sup>, no known fructose intolerance, no medical illnesses, and no use of medication. The subjects were informed orally and in writing about potential risks and discomforts associated with the study. Screening of potential subjects included medical history, physical examination, and standard baseline laboratory tests. The study protocol was approved by the Ethics Committee of the Capital Region of Denmark (H-18000074) in accordance with the Helsinki Declaration. All subjects provided written informed consent to participation.

### Experimental Design

The study consisted of 3 trials: (1) fructose ingestion plus infusion of somatostatin; (2) fructose ingestion plus infusion of saline; and (3) water ingestion and infusion of somatostatin. Infusion of somatostatin was used to reduce the concentration of circulating insulin in the trials. The subjects participated in the 3 trials in randomized order on 3 different days separated by at least 1 week. All experiments were performed in the morning after an overnight fast (from 10 PM the previous evening) and the subjects remained fasting throughout the trials but had free access to water. The duration of the trials was 6 hours and 30 minutes. The subjects rested in a supine position during the trials. An intravenous catheter was inserted into the left and right antecubital vein, one used for infusion of

somatostatin or saline and one for collecting blood samples. In the fructose + somatostatin trial, a 15-minute rest was followed by a 15-minute infusion of somatostatin (Octreotide, Hospira Nordic AB, Stockholm, Sweden) at 100 ng/kg/min. Afterwards, 75 g of fructose dissolved in 250 mL of water was ingested (minute 0). In the fructose trial, after a 15-minute rest, saline was infused (100 ng/kg/min) over 15 minutes followed by ingestion of 75 g of fructose dissolved in 250 mL of water (minute 0). In the somatostatin trial, after a 15-minute rest, the subjects received a 15-minute infusion of somatostatin (100 ng/kg/min) followed by ingestion of 250 mL of water (minute 0). In the trials where fructose was ingested, 2 subjects experienced gastrointestinal symptoms (diarrhea or flatulence) for 30 to 180 minutes following the oral fructose ingestion. There appears to be no link between these symptoms and the results of the study. Blood samples were obtained every 30 minutes during the first 2 hours and 30 minutes, and afterwards every hour for the last 4 hours.

### Plasma Analysis

All plasma samples were analyzed at the Department of Clinical Biochemistry, Rigshospitalet. Standard baseline laboratory tests were analyzed according to standard procedures. During the trials, the blood samples were collected in EDTA tubes and centrifuged at 4 °C at 3100g for 15 minutes. The plasma fractions were stored at -80 °C until analyzed. Plasma triacylglycerol, uric acid, glucose, lactate, and fatty acids were analyzed by enzymatic colorimetric assays (Cobas 8000, Roche and Wako NEFA-HR [2]). Plasma insulin and C-peptide were analyzed by electrochemiluminescent immunoassays (Cobas 8000, Roche). The lowest detection limit for insulin was 7 pmol/L. Insulin levels measured lower than 7 pmol/L were set to 7 pmol/L. Plasma glucagon was analyzed by an enzyme-linked immunosorbent assay (ELISA) (Mercodia). The lowest detection limit for glucagon was 1.37 pmol/L and glucagon levels below the detection limit were set to 1.37 pmol/L. Plasma FGF21 and follistatin were analyzed by an ELISA kit (R&D Systems), with detection limits of 31.3 ng/L and 250 ng/L, respectively. Plasma ANGPTL4 and GDF15 were analyzed by an ELISA kit from BioVendor and R&D Systems, respectively, with detection limits 0.173 µg/L and 23.4 ng/L, respectively. All samples were run in duplicate for the analysis of plasma glucagon, FGF21, follistatin, ANGPTL4, and GDF15. For the plasma FGF21 analysis, the FGF21 levels in 1 subject were below the lowest detection limit in all 3 trials and these results were therefore excluded from the FGF21 results. For the plasma ANGPTL4 analysis, 2 subjects had extremely elevated plasma levels in all 3 trials (increased by 20- to 40-fold compared with the

other subjects). A re-analysis of the plasma samples yielded the same elevated plasma levels and these levels were therefore excluded from the ANGPTL4 results.

### Statistical Analysis

Data are presented as means ± standard error of the mean. Statistical analyses were performed in SAS 9.4 (SAS Institute Inc). For comparisons between the trials, a mixed model analysis of variance (ANOVA) with repeated measurements and baseline values as a covariate (ANCOVA) was used. Fixed effects in the model were group and time. For comparison between time points in each trial, a mixed model ANOVA with repeated measurements was used. When the ANOVAs revealed significant differences, Tukey's post hoc test was applied for multiple comparisons.  $P < 0.05$  was considered statistically significant.

## Results

### Subject Characteristics

Ten healthy male subjects were included in the study and all subjects participated for the 3 experimental days (fructose; fructose + somatostatin; somatostatin). Clinical data of the subjects are summarized in Table 1. In the study, 2 subjects with a BMI of 26.1 and 26.5 kg/m<sup>2</sup> were included, since it was expected it would not affect the outcome of the study. Apart from 2 subjects with LDL cholesterol of 3.1 and 3.4 mmol/L, all values were within the normal range, indicating no metabolic or liver diseases. There was no correlation between subjects with BMI > 25 kg/m<sup>2</sup> and LDL > 3.0 mmol/L.

### The Hepatokine Response to Fructose Ingestion

Circulating FGF21, follistatin, ANGPTL4, and GDF15 were evaluated in response to ingestion of 75 g of fructose in combination with infusion of somatostatin. Plasma FGF21 [Fig. 1A] concentrations peaked at 120 minutes in all 3 trials. Following fructose ingestion (fructose trial), plasma FGF21 increased and peaked with a 3.7-fold increase (~292 ng/L) ( $P < 0.05$ ) above baseline. Ingestion of fructose combined with the somatostatin infusion (fructose + somatostatin trial) resulted in a further increase in plasma FGF21 with a 4.9-fold increase (~515 ng/L) in peak concentration ( $P < 0.05$ ) and increased levels from minute 120 to 300 compared with fructose ingestion only. Infusion of somatostatin alone (somatostatin trial) resulted in a small 1.4-fold increase (~171 ng/L) in plasma FGF21 ( $P < 0.05$ ).

Plasma follistatin concentration [Fig. 1B] in the fructose trial tended to decrease in the middle of the trial from

**Table 1.** Clinical data—nonfasting venous blood samples at screening (n = 10).

Clinical data	Values	Reference values
Body mass index (kg/m <sup>2</sup> )	22.9 ± 2.4	18.5-25
Systolic blood pressure (mm Hg)	124.6 ± 6.4	< 140
Diastolic blood pressure (mm Hg)	75.3 ± 9.1	< 90
<b>Red blood cells:</b>		
Hemoglobin (mmol/L)	8.9 ± 0.7	8.3-10.5
<b>Inflammation:</b>		
Leucocytes (x10 <sup>9</sup> /L)	5.7 ± 1.2	3.5-8.8
hsCRP (mg/L)	1.5 ± 0.5	< 3.0
<b>Kidney:</b>		
Sodium (mmol/L)	142 ± 1.4	137-144
Potassium (mmol/L)	4.0 ± 0.2	3.5-4.4
Creatinine (umol/L)	83 ± 6.2	60-105
<b>Glucose levels:</b>		
Hemoglobin A1c (mmol/mol)	32.7 ± 2.0	< 48
<b>Lipids:</b>		
Total cholesterol (mmol/L)	4.3 ± 0.6	< 5.0
HDL cholesterol (mmol/L)	1.5 ± 0.2	> 1.0
LDL cholesterol (mmol/L)	2.7 ± 0.5	< 3.0
Triacylglycerol (mmol/L)	1.0 ± 0.3	< 2.0
<b>Liver characteristics:</b>		
Albumin (g/L)	43.9 ± 2.0	36-48
INR	1.1 ± 0.1	< 1.2
Aspartate transaminase (U/L)	26.8 ± 4.5	15-45
Alanine transaminase (U/L)	24.6 ± 6.2	10-70
Alkaline phosphatase (U/L)	78.8 ± 25.1	35-105
Gamma-glutamyl-transferase (U/L)	18.7 ± 5.7	10-80

Values are means ± SD.

Abbreviations: HDL, high-density lipoprotein; hsCRP, high sensitivity C-reactive protein; INR, international normalized ratio; LDL, low-density lipoprotein.

minute 180 to 240 ( $P = 0.06$ ) before it increased 1.8-fold (~2210 ng/L) above baseline at minute 360 ( $P < 0.05$ ). The fructose + somatostatin trial resulted in decreased levels of plasma follistatin from minute 300 while somatostatin alone resulted in an approximately 1.5-fold increase (~1540 ng/L) from minute 180.

Plasma ANGPTL4 [Fig. 1C] decreased modestly during the fructose trial from minute 120 to 240, whereas no differences were observed during the fructose + somatostatin and somatostatin trials. The plasma ANGPTL4 level during the somatostatin trial was higher compared with the fructose trial from minute 180 to 240. For plasma GDF15 [Fig. 1D], no differences were observed during any of the trials.

### Impact of Fructose Ingestion on Glucose Metabolism

Next, we evaluated the effect of fructose ingestion and somatostatin infusion on glucose metabolism.

Plasma insulin [Fig. 2A] peaked with a 2.5-fold increase (~118 pmol/L) ( $P < 0.05$ ) during the fructose trial at minute 30 following fructose ingestion ( $P < 0.05$ ) and was elevated until minute 90. During the fructose + somatostatin trial, insulin initially decreased from minute 0 to 90 but returned to baseline when plasma glucose peaked. Plasma insulin levels during the fructose trial were higher than the fructose + somatostatin trial from minute 0 to 120. During the somatostatin trial, plasma insulin was decreased at minute 0 and remained decreased during the whole trial. The kinetics for plasma C-peptide [Fig. 2B] concentration during the 3 trials mirrored those of plasma insulin. During the fructose trial, plasma glucagon [Fig. 2C] was initially decreased from minute 0 to 120 before returning to baseline. This was followed by a decrease in concentration from minute 300 to 360. The largest increase in plasma glucose [Fig. 2D] was obtained in the fructose + somatostatin trial, with increased levels from minute 60 to 180 and a 1.7-fold increase (~8.8 mmol/L) at minute 120 ( $P < 0.05$ ).

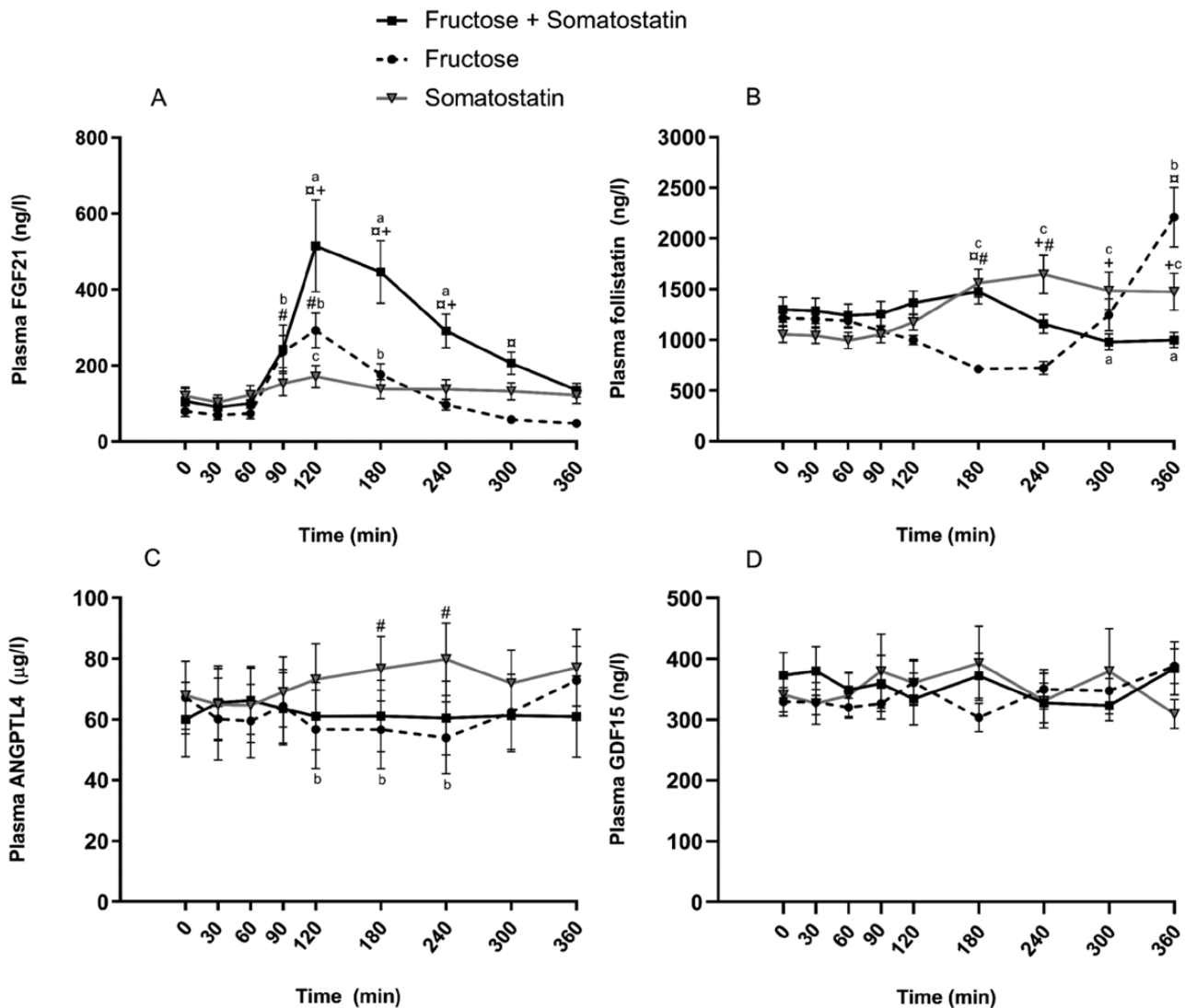
### Impact of Fructose Ingestion on Selected Metabolites

Following fructose ingestion, plasma lactate [Fig. 3A] and plasma uric acid [Fig. 3B] were increased in the fructose + somatostatin trial and the fructose trial. Plasma fatty acid [Fig. 3C] concentration in the trials changed in inverse ratio to plasma insulin levels. Thus, the highest concentrations were observed in the somatostatin trial. Plasma triacylglycerol [Fig. 3D] in the fructose trial was decreased from minute 60 to 120 and tended to increase at minute 360 ( $P = 0.07$ ) while it was decreased in the fructose + somatostatin trial from minute 120 to 360 and in the somatostatin trial from minute 240 to 360.

### Discussion

The aim of the present study was to investigate the acute effect of insulin levels and fructose ingestion on circulating levels of hepatokines. Here, we show in humans (1) that the fructose-induced increase in plasma FGF21 is enhanced when pretreated with somatostatin; (2) furthermore, that fructose ingestion also increased plasma follistatin concentration, but the increase was delayed compared with the increase in plasma FGF21; and (3) in contrast, fructose ingestion had no effect on ANGPTL4 and GDF15 levels, demonstrating differences in the response to fructose ingestion between FGF21, follistatin, ANGPTL4, and GDF15 in humans.

Fructose-induced FGF21 has previously been demonstrated in humans [6, 27, 35]. The present study confirms the finding that fructose ingestion increases plasma FGF21.



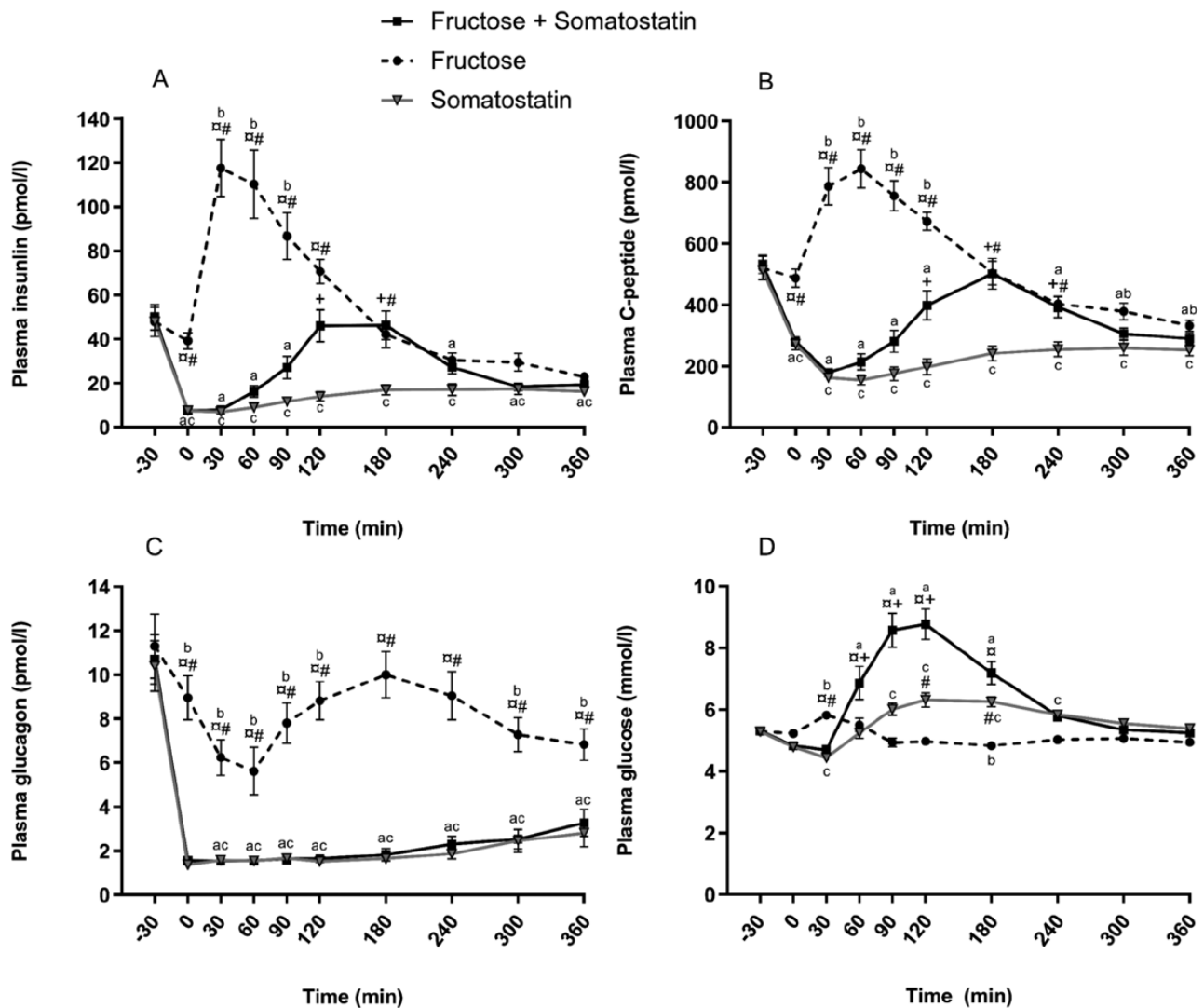
**Figure 1.** Plasma response of FGF21 ( $n = 9$ ), follistatin ( $n = 10$ ), ANGPTL4 ( $n = 8$ ), and GDF15 ( $n = 10$ ) to fructose and somatostatin in healthy young men. Effects of fructose ingestion combined with 15 minutes of somatostatin infusion (■, black line), fructose ingestion (●, dashed line) or 15 minutes infusion of somatostatin (▼, gray line) on A, plasma FGF21; B, plasma follistatin; C, plasma ANGPTL4; and D, GDF15. Somatostatin was infused from minute  $-15$  to  $0$ . Fructose was ingested at minute  $0$ . Data are presented as means  $\pm$  SEM. Statistical significance is marked by: a, effect of time for fructose + somatostatin; b, effect of time for fructose; c, effect of time for somatostatin;  $\square$ , between fructose + somatostatin and fructose; +, between fructose + somatostatin and somatostatin; #, between fructose and somatostatin.  $P < 0.05$  was considered statistically significant.

Fructose does not stimulate insulin from beta cells [36, 37] and the plasma insulin increase observed in the present study following fructose ingestion is mediated by an increase in plasma glucose converted from the ingested fructose either in the gut or liver. When fructose is ingested in combination with somatostatin, the plasma glucose level is augmented, demonstrating that the modest increase in plasma insulin has marked effect on hepatic glucose metabolism.

Ingestion of 75 g of glucose has also been shown to increase circulating FGF21 [6, 38] and when compared with fructose ingestion, the FGF21 response from glucose ingestion was reduced and delayed with an increased

circulating insulin response [6]. Glucose per se does not regulate FGF21, as an acute elevation of plasma glucose concentration during low insulin concentrations does not affect circulating FGF21 in healthy volunteers [38]. This suggests that the increase in circulating FGF21 during the fructose + somatostatin trial is not a result of increased glucose levels during the trial.

A regulatory pathway for FGF21 in the liver is activation of the transcription factor carbohydrate-responsive element binding protein (ChREBP) [39, 40]. In response to fructose ingestion, it has been shown that ChREBP is necessary for increased hepatic mRNA expression and circulating levels of FGF21 in rodents [41]. Besides fructose,

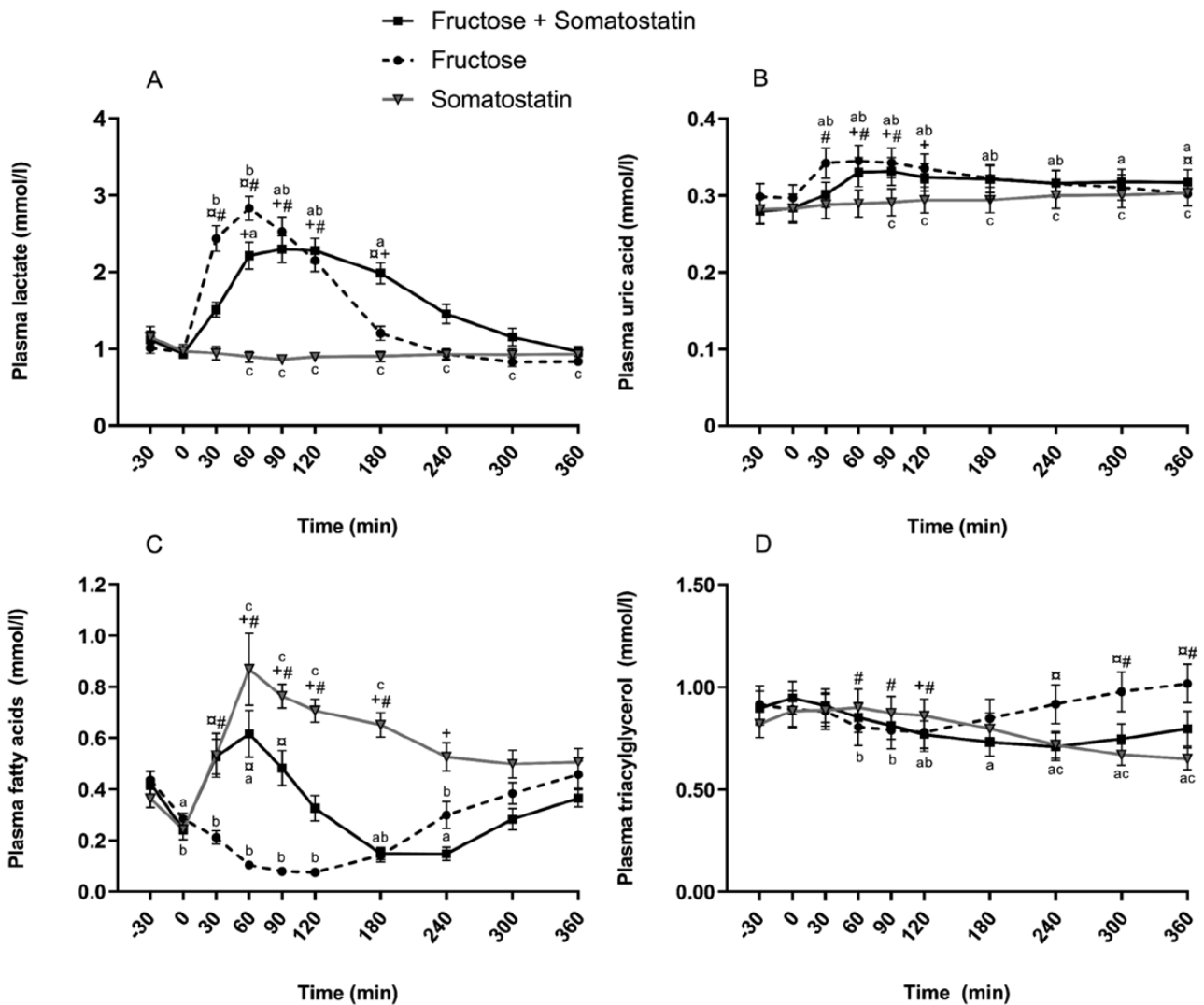


**Figure 2.** Impact of fructose ingestion and somatostatin on glucose metabolism in healthy young men. Effects of fructose ingestion combined with 15 minutes of somatostatin infusion (■, black line), fructose ingestion (●, dashed line) or 15 minutes infusion of somatostatin (▼, gray line) on A, plasma insulin; B, plasma C-peptide; C, plasma glucagon; and D, plasma glucose. Somatostatin was infused from minute -15 to 0. Fructose was ingested at minute 0. Data are presented as means  $\pm$  SEM. Statistical significance is marked as: a, effect of time for fructose + somatostatin; b, effect of time for fructose; c, effect of time for somatostatin;  $\square$ , between fructose + somatostatin and fructose; +, between fructose + somatostatin and somatostatin; #, between fructose and somatostatin.  $P < 0.05$  was considered statistically significant.

glucagon also regulates circulating FGF21 levels in humans. An acute (hours) increase in plasma FGF21 occurs following an increase in plasma glucagon when administered pharmacologically (intramuscular or intravenous) [25, 42] or when increased physiologically during an acute bout of exercise [43], with some studies supporting the response of circulating FGF21 by glucagon is inhibited by circulating insulin levels [44, 45]. Stimulation of hepatic glucagon receptors activates adenylyl cyclase and increases intracellular levels of cyclic adenosine monophosphate (cAMP), while insulin reduces cAMP levels via the activation of phosphodiesterase [46, 47]. Therefore, the level of hepatic cAMP is a result of the combined glucagon and insulin signaling. A reduced action of insulin on the liver,

as observed during insulin resistance, could attribute to the elevated FGF21 levels in patients with metabolic syndrome and in patients with type 2 diabetes when compared to matched control subjects [48].

In the present study, when pretreated with a 15-minute somatostatin infusion, the fructose-induced increase in plasma FGF21 is enhanced. A potential explanation for the increase in circulating FGF21 in the somatostatin + fructose trial could be the decreased plasma insulin levels during the trial, mimicking the effect of insulin on glucagon-induced FGF21. The inhibitory effect of circulating insulin on fructose-induced FGF21 could explain previous studies, where fructose-induced FGF21 was increased in patients with the metabolic syndrome compared with healthy



**Figure 3.** Plasma lactate, uric acid, fatty acids, and triacylglycerol in response to fructose ingestion and somatostatin infusion in healthy young men. Effects of fructose ingestion combined with 15 minutes of somatostatin infusion (■, black line), fructose ingestion (●, dashed line), or 15 minutes infusion of somatostatin (▽, gray line) on A, plasma lactate; B, plasma uric acid; C, plasma fatty acids; and D, plasma triacylglycerol. Somatostatin was infused from minute -15 to 0. Fructose was ingested at minute 0. Data are presented as means  $\pm$  SEM. Statistical significance is marked as: a, effect of time for fructose + somatostatin; b, effect of time for fructose; c, effect of time for somatostatin;  $\square$ , between fructose + somatostatin and fructose; +, between fructose + somatostatin and somatostatin; #, between fructose and somatostatin.  $P < 0.05$  was considered statistically significant.

subjects [6, 27]. Whether a potential inhibitory effect of insulin on fructose-induced FGF21 is through a fructose-induced cAMP increase is currently not known, as only 1 in vitro study has reported a link between fructose and increased intrahepatic cAMP levels [49]. Likewise, somatostatin inhibits the secretion of several hormones, including insulin, glucagon, and multiple gastrointestinal hormones, which also could affect the outcome.

However, contrary to the above, hours of hyperinsulinemia increase circulating FGF21 during euglycemia. Several studies have demonstrated an increase in circulating FGF21 concentrations after insulin stimulation during a hyperinsulinemic-euglycemic clamp, suggesting that insulin has a stimulatory effect on circulatory

FGF21 when elevated for several hours [38, 50-52]. In one study comparing pancreatic clamps, it was shown that insulin infusion, and not glucose infusion, was required for increasing circulating FGF21 in young healthy males [38]. In contrast to the metabolism of glucose, fructose metabolism occurs independently of insulin and there is no negative feedback by ATP in the hepatocyte [53] suggesting a complex interaction between circulating insulin and circulating FGF21 concentrations in response to carbohydrate ingestion.

Nevertheless, other regulatory pathways could act in concert with insulin. Fatty acids have also been shown to be a stimulator for FGF21 via PPAR $\alpha$  [54]. In the present study, plasma fatty acids during the fructose + somatostatin

and somatostatin trial were increased. However, it is unlikely that the increased levels of fatty acids would affect plasma FGF21 in this study, since it required 240 minutes of elevated circulating fatty acid levels before a 1.3-fold increase in circulating FGF21 was measured in humans [55], which differs from the present study as peak concentration of plasma FGF21 was obtained after 120 minutes in all 3 trials. However, recent findings in mice show that fructose is converted to acetate by the gut microbiome contributing to hepatic de novo lipogenesis [56]. Fructose ingestion could, through the gut-liver cross-talk with acetate, potentially stimulate plasma FGF21 concentrations well before the rise in circulating levels of fatty acids in the present study. Whether the fructose-stimulating signal is via ChREBP, PPAR $\alpha$ , cAMP, or a yet unidentified mechanism must be established.

The effects of fructose on circulating follistatin have not been investigated previously. The present study revealed that fructose ingestion increases circulating follistatin with a delay of 4 hours compared with FGF21, and surprisingly, only in the absence of somatostatin. Lactate and uric acid levels increase following fructose ingestion in humans [53, 57-59]. In both the fructose + somatostatin and the fructose trial, an increase is observed in circulating lactate and uric acid levels; however, the delayed peak concentration of plasma lactate and uric acid in the fructose + somatostatin trial compared to the fructose trial suggests a 30-minute delay in fructose absorption. Delayed gastric emptying is a common side effect of somatostatin infusion. A delay in absorption of fructose could in theory also delay the increase in plasma follistatin to appear after the last timepoint (360 minutes) in the fructose trial.

Plasma concentration of glucagon increases, and plasma concentration of insulin reduces the circulating level of follistatin in young healthy men [11] and patients with type 2 diabetes have elevated circulating follistatin levels [20]. A recent study by Tao et al (2018) showed that follistatin is regulated by the transcription factor FoxO1 in the liver [26]. FoxO1 activates gluconeogenic gene expression and is inhibited by insulin through the PI3K-AKT cascade [60]. In the present study, plasma follistatin tended to decrease from minute 180 to 240 following fructose ingestion, which is preceded by an insulin peak at minute 30 to 90, supporting an inhibitory effect of circulating insulin on plasma follistatin. In mice, it has been shown that administration of fructose increased hepatic mRNA of FoxO1, however it also simultaneously increased phosphorylation of FoxO1 [61].

A recent study has shown that glucagon, through cAMP and PKA, also regulates FoxO1 by promoting nuclear translocation and stability of FoxO1 [62], suggesting opposite regulatory effects of insulin and glucagon/cAMP. In

the present study, a rise in the plasma glucagon concentration from minute 60 to 180 in the fructose trial could explain the increase in plasma follistatin at minute 360. This could also explain why no increase in plasma follistatin was observed in the fructose + somatostatin trial, as the glucagon concentration was reduced throughout the whole trial. The late increase in plasma follistatin following fructose ingestion could also be secondary to one of many stimuli blunted by the somatostatin infusion.

Whether the increase in circulating follistatin following fructose ingestion in the present study is caused by increased circulating glucagon, by fructose-induced hepatic FoxO1 mRNA, or a yet unknown pathway is not fully understood. This needs to be investigated further.

Interestingly, in contrast to FGF21 and follistatin, circulating ANGPTL4 levels were reduced following fructose ingestion in the present study. Like FGF21 and follistatin, infusion of glucagon increases circulating ANGPTL4, and elevation of cAMP levels in hepG2 cells increases ANGPTL4 mRNA levels [12]. Hepatic gene expression of ANGPTL4 is regulated by several PPARs, including PPAR $\alpha$  [63], and cAMP has been shown to regulate the activity of different PPARs [64]. Since fructose ingestion does not increase plasma ANGPTL4 in the present study, it suggests that the increase in FGF21 and follistatin does not signal through cAMP. Infusion of somatostatin alone has previously showed increased plasma ANGPTL4 levels [12] and both plasma FGF21 and follistatin were increased following somatostatin infusion in the present study. This could suggest a common regulatory mechanism for the 3 hepatokines. However, no increase in plasma ANGPTL4 was observed following somatostatin infusion in the present study. This needs to be investigated further. ANGPTL4 is an inhibitor of lipoprotein lipase, which hydrolyzes plasma triacylglycerols in adipose tissue [65]. In the fructose trial, decreases in both plasma ANGPTL4 and triacylglycerols were observed; however, the decrease in plasma triacylglycerols preceded the decrease in plasma ANGPTL4.

We included GDF15 in the study as GDF15 shares regulatory similarities with FGF21 and it is proposed that the GDF15 origin is either from the liver [33] or the intestine [34]. In response to fructose ingestion and/or somatostatin, no increase in circulating GDF15 levels were measured.

In conclusion, the present study demonstrates the effect of fructose ingestion on circulating FGF21 levels in humans and it is proposed that there is an inhibitory effect of insulin on fructose-induced FGF21 levels. It is further demonstrated that fructose ingestion increases circulating follistatin 6 hours after ingestion, while no increase was measured for circulating ANGPTL4 or GDF15. Glucagon can acutely increase circulating FGF21, follistatin, and ANGPTL4 in



humans; however, this study highlights that the regulation is far more complex in humans, suggesting other molecular pathways for fructose metabolism to increase hepatokine production. How fructose ingestion affects the circulating levels of hepatokines—especially in conditions with insulin resistance—is not fully understood. While there is a possible effect of insulin on fructose-induced FGF21, the effect on fructose-induced follistatin seems absent, even though earlier studies have shown that the basal circulating level of follistatin is increased in conditions with insulin resistance. Further studies are required to describe the regulatory mechanisms of hepatokines in humans.

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## Additional Information

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**Disclosures:** The authors have declared that no conflict of interest exists.

**Data Availability:** Datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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